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Sialylation acts as a checkpoint for innate immune responses in the central nervous system

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Abstract

Sialic acids are monosaccharides that normally terminate the glycan chains of cell surface glyco-proteins and -lipids in mammals, and are highly enriched in the central nervous tissue. Sialic acids are conjugated to proteins and lipids (termed "sialylation") by specific sialyltransferases, and are removed ("desialylation") by neuraminidases. Cell surface sialic acids are sensed by complement factor H (FH) to inhibit complement activation or by sialic acid-binding immunoglobulin-like lectin (SIGLEC) receptors to inhibit microglial activation, phagocytosis, and oxidative burst. In contrast, desialylation of cells enables binding of the opsonins C1, calreticulin, galectin-3, and collectins, stimulating phagocytosis of such cells. Hypersialylation is used by bacteria and cancers as camouflage to escape immune recognition, while polysialylation of neurons protects synapses and neurogenesis. Insufficient lysosomal cleavage of sialylated molecules can lead to lysosomal accumulation of lipids and aggregated proteins, which if excessive may be expelled into the extracellular space. On the other hand, desialylation of immune receptors can activate them or trigger removal of proteins. Loss of inhibitory SIGLECs or FH triggers reduced clearance of aggregates, oxidative brain damage and complement-mediated retinal damage. Thus, cell surface sialylation recognized by FH, SIGLEC, and other immune-related receptors acts as a major checkpoint inhibitor of innate immune responses in the central nervous system, while excessive cleavage of sialic acid residues and consequently removing this checkpoint inhibitor may trigger lipid accumulation, protein aggregation, inflammation, and neurodegeneration.

KEYWORDS
complement system, desialylation, microglia, neuraminidases, sialic acid, SIGLEC

1 | THE INNATE IMMUNE RESPONSE OF THE BRAIN

The blood brain barrier normally restricts access of antibodies and white blood cells into the central nervous system (CNS, i.e., brain, spinal cord, and retina). Thus, under homeostatic conditions, the adaptive immune response of the CNS is very limited, and the innate immune response depends on endogenous brain cells, in particular the microglia. Microglia are the main phagocytes of the CNS. In the healthy brain, the innate immune response of microglia is turned "OFF," a state that has been termed "resting" or "homeostatic." However, even in this state, microglial processes are highly active and

continuously surveying the CNS for danger in the form of pathogens or damage (Nimmerjahn, Kirchhoff, & Helmchen, 2005). Upon encounter of danger signals, the innate immune response is turned "ON," and the microglia switch into various "activated" phenotypes. Activated microglia may: (a) retract their processes and the whole cell becomes motile, (b) release reactive oxygen and nitrogen species to kill pathogens, (c) release chemokines and cytokines attracting and activating other immune cells (e.g., monocytes and T lymphocytes), and (d) become highly phagocytic, to remove pathogens, dead or dying neurons and clear up debris. However, excessive phagocytosis or inflammation may contribute to the pathogenesis of stroke, brain trauma, epilepsy, brain infections, schizophrenia, and neurodegenerative disease (such as Alzheimer's disease [AD]), emphasizing the importance of understanding microglial activation (Vilalta & Brown, 2018).

Microglial activation, and thus the innate immune response, is turned "ON" by stimulation of pattern recognition receptors (such as the toll-like receptors) or other cell surface receptors sensing the presence of pathogens or damage. On the other side, microglial activation, and the innate immune response generally, can cause collateral damage to nonregenerative neurons, and thus, has strong "OFF" switches that act as innate immune checkpoints. Sialic acid residues on the surface of brain cells are molecules signaling the presence of "intact self" and act as such an "OFF" switch and checkpoint suppressing innate immunity. Consequently, sialic acid residues can prevent microglial attack of intact self. Here, we review the current literature on how these sialic acid residues suppress and regulate the innate immune system of the CNS, in particular microglia, and what happens when this regulatory switch of the immune response is removed.

2 | SIALIC ACIDS

Sialic acid (Sia) is a generic term for a family of nine carbon monosaccharides, that are derivatives of neuraminic acid. Sia is the typical residue terminating the sugar chains of glycolipids and glycoproteins expressed on the cell surface of vertebrates (T. Angata & Varki, 2002; Varki, 2017). There are three main types of Sias in vertebrates, namely *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and deaminoneuraminic acid (Kdn). Most mammals produce Neu5Ac and Neu5Gc, while humans have lost the capacity to produce Neu5Gc (Varki, 2017). Sias can be modified by several postsynthesis modifications, such as methylation, sulfation, lactylation, acetylation, and lactonization and can be added to sugar chains, a process named "sialylation," to form sialoglycoproteins and sialoglycolipids. Thus, the Sia family consists of more than 50 members with different structures (T. Angata & Varki, 2002).

The biosynthesis of Sia takes place in the cytosol and involves four steps and three enzymes (Figure 1). Two rate-limiting steps are carried out by the bifunctional glucosamine (UDP-*N*-acetyl)-2-epimerase/*N*-acetylmannosamine kinase (GNE; Keppler, 1999). GNE catalyses the conversion from uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) to *N*-acetyl-D-mannosamine (ManNAc), and phosphorylates ManNAc to form *N*-acetyl-mannosamine 6-phosphate (ManNAc-6-P). ManNAc-6-P is then condensed and dephosphorylated in two steps to generate Neu5Ac. In the nucleus, Neu5Ac is conjugated by the cytidine monophosphate *N*-acetylneuraminic acid synthetase (CMAS; Munster-Kuhnel et al., 2004) with cytidine 5-monophosphate (CMP) to form the active CMP-Neu5Ac. After transport of CMP-Neu5Ac from the nucleus to the Golgi apparatus, sialyltransferases conjugate Neu5Ac to a galactose (Gal), an *N*-acetylgalactosamine (GalNAc), or another Sia residue of various proteins or lipids (Figure 1; Chen & Varki, 2010). Humans have approximately 20 different sialyltransferases that add Sia to different acceptor sugars (Gal, GalNAc, Sia) by using distinct linkages (α2,3-, α2,6-, α2,8-) between the Sia residues and the underlying acceptor sugar. Moreover, Sias can form homopolymers with different degrees of polymerization via intersialyl linkages. For instance, polysialic acid (polySia) is a homopolymer of α2,8-linked Neu5Ac monomers with a degree of polymerization between 10 and around 200, and is highly expressed in the developing CNS (Schnaar, Gerardy-Schahn, & Hildebrandt, 2014).

Sias are detected on most cell surfaces and secreted molecules (Figure 2). Especially nervous system and immune cells show a very high abundance and diversity of sialoglycoconjugates on their cell surface. For example, the concentration of Sias within the glycocalyx of lymphocytes has been estimated to be 110 mM (Collins et al., 2004), while overall, the CNS has the highest concentration of Sias in the body (Schnaar et al., 2014). However, knowledge on the exact Sia levels of different human brain cells is incomplete. In general, the glycosylation pattern of the glycocalyx regulates various functions within the local microenvironment, such as the folding, turnover, trafficking, and signaling of proteins within the cell membrane (as reviewed in Liao, Klaus, & Neumann, 2020) and impaired glycosylation can lead to neurodegenerative diseases (see Box 1). The specific function of each glycan is usually determined by the outermost sugars, which in mammals is mainly Sia.

Box 1. Sialylation as checkpoint in Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disease and leads to dementia in the elderly people. AD is characterized by memory loss and difficulties with thinking, problem solving and language and ranges in severity from mild to later very severe stages. The main neuropathological features are extracellular deposits of amyloid-β (Aβ), intraneuronal tangles composed of hyperphosphorylated forms of the tau protein and dysmorphic microglia. Genome-wide association studies have highlighted microglial genes including *SIGLEC-3* (Hollingworth et al., 2011; Malik et al., 2013; Naj et al., 2011) and *SIGLEC-11* (Bellenguez et al., 2020) as well as components of the complement system such as *CR1* (Harold et al., 2009; Hollingworth et al., 2011; Lambert et al., 2009), and *FH* (Zhang et al., 2016) among the major risk factors of AD. In parallel, more and more evidence arises that sialylation plays an important role in AD. Protein studies on cerebrospinal fluid (Hajjar, Liu, Jones, & Uppal, 2020), serum (Maguire et al., 1994), and postmortem brain tissue (Maguire & Breen, 1995) of AD patients have revealed

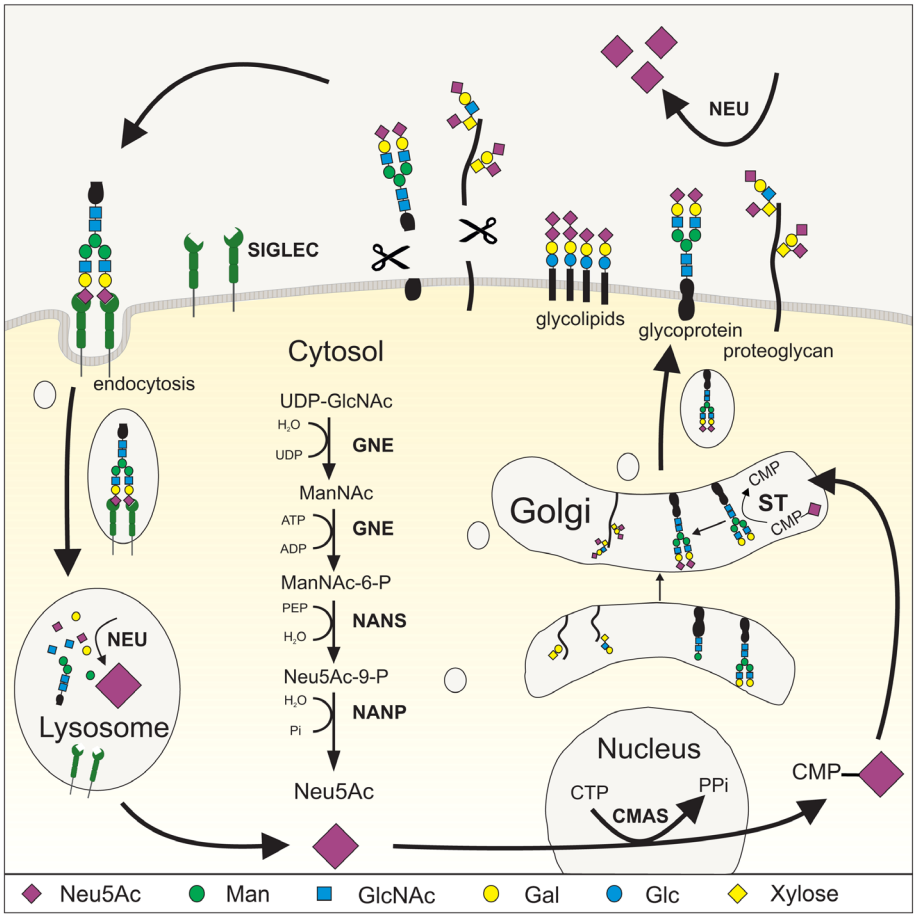

FIGURE 1 The sialic acid cycle from production, to cell surface expression, removal by desialylation, uptake, lysosomal digestion and reuse. *N*-Acetylneuraminic acid (Neu5Ac) can be synthesized de novo in the cytosol from uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) in four different reactions. The first two rate-limiting steps are catalyzed by the glucosamine (UDP-*N*-acetyl)-2-epimerase/N-acetylmannosamine kinase (GNE). The following steps are mediated by *N*-acetylneuraminic acid synthase (NANS) and *N*-acetylneuraminic acid phosphatase (NANP). In the nucleus Neu5Ac is activated with a cytidine monophosphate residue (CMP) by the CMP-sialic acid synthetase (CMAS). Activated Neu5Ac gets transported into the Golgi apparatus. Here, sialoglycoconjugates are formed by the transfer of the activated sialic acid molecule onto a glycoconjugate by a sialyltransferase (ST). Glycoconjugates are then transported to their target membrane to form the glycocalyx. On the glycocalyx, either the terminal sialic acid residues of sialylated glycostructures can be cleaved by neuraminidases (NEU) or the complete sialylated glycoconjugate can be shed. While sialylated GPI-anchored proteins are cleaved by phospholipases, the sialylated transmembrane proteins are cleaved by metalloproteinases (scissor). Soluble cleaved or shed sialylated glycostructures or sialooligosaccharides are recognized by sialic acid-binding immunoglobulin-like lectin (SIGLEC) that can trigger cellular uptake of the structures by endocytosis. Thus, Neu5Ac of sialoglycoconjugates can be recycled via the endosomal-lysosomal pathway. NEU can cleave off Neu5Ac for reuse in the sialic acid cycle. ADP, adenosine diphosphate; ATP, adenosine triphosphate; CMAS, CMP-sialic acid synthethase; CMP, cytidine monophosphate; CTP, cytidine triphosphate; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GNE, glucosamine-2-epimerase/*N*-acetylmannosamine kinase; Man, mannose; ManNAc, *N*-acetylmannosamine; NANP, *N*-acetylneuraminic acid phosphatase; NANS, *N*-acetylneuraminic acid synthase; NEU, neuraminidase; Neu5Ac, *N*-acetylneuraminic acid; PEP, phosphoenolpyruvic acid; Pi (or PPi), activated phosphate(s); SIGLEC, sialic acid-binding immunoglobulin-like lectin; ST, sialyltransferase; UDP, uridine diphosphate

decreased protein sialylation and a decrease in enzymes responsible for protein sialylation.

Sialylation serves as a checkpoint that can protect the removal of cellular structures like synapses and neurons. An impaired or insufficient sialylation enables aggregation of proteins like Aβ and phosphorylated tau as seen in the disease-affected muscles of patients diagnosed with GNE myopathy (Argov & Yarom, 1984; Murakami, Ihara, & Nonaka, 1995; Muth, Barthel, Bähr, Dalakas, & Schmidt, 2009; J. Schmidt et al., 2012). Furthermore, loss of Sia or desialylation is seen as a molecular sign of protein aging (Grozovsky et al., 2014; Li et al., 2015; Mehdi, Singh, & Rizvi, 2012; Qiu et al., 2016; Yang et al., 2015), and proper desialylation seems to be essential for a correct degradation of the amyloid protein precursor (APP; Annunziata et al., 2013) and thus, for prevention of Aβ accumulation in the cell.

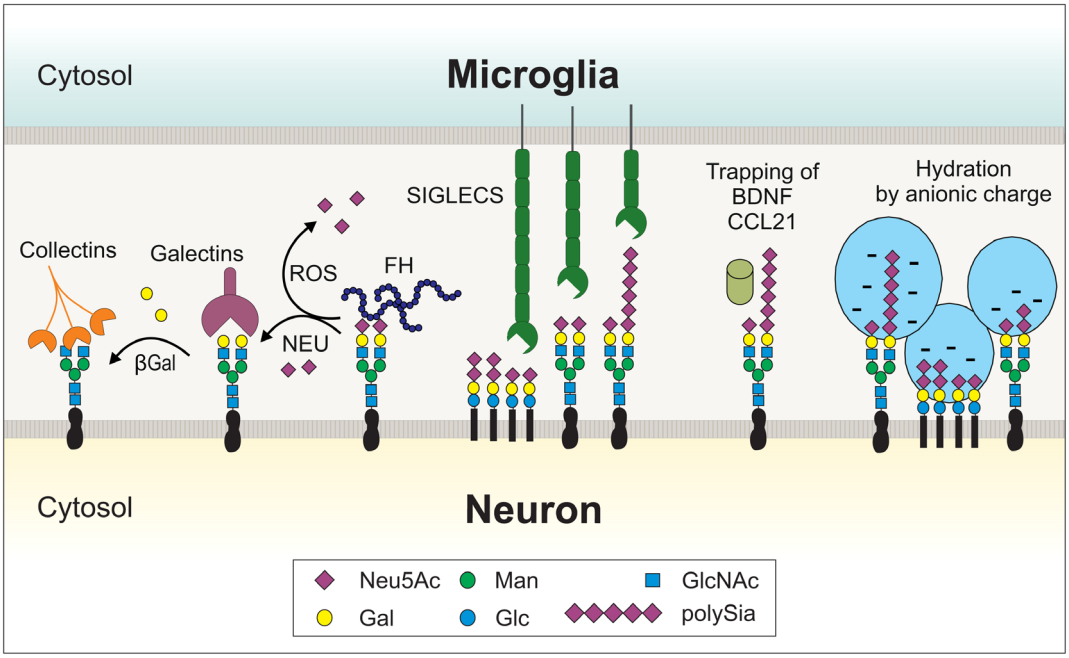

FIGURE 2 Sialic acids (Sias) play a unique role in the glycosylation pattern of the cellular glycocalyx. The sugar chains of cell surface glycoproteins and glycolipids, together with crosslinking proteoglycans, are collectively known as the glycocalyx. The diverse glycosylation pattern of the glycocalyx can fulfill a variety of functions within the local environment, such as protein folding and protein stability within the cellular membrane, protein trafficking, or protein signaling. Due to the unique position of sialic acid residues as terminal caps of glyco-proteins and -lipids and their negative charge, Sias can form a hydrophilic reservoir that can trap soluble factors like the locally produced brain-derived neurotrophic factor (BDNF) or the chemokine CCL21. Furthermore, Sias (such as Neu5Ac) can be recognized by receptors (e.g., sialic acid-binding immunoglobulin-like lectin, SIGLEC), proteins (e.g., complement factor H, FH) or be involved as ligands in cell–cell interaction. Alternatively, Sias can be removed by neuraminidase (NEU), enabling binding of galectin-3 and other proteins to the exposed galactose (Gal) residues. In turn, Gal can be removed by beta galactosidase (βGal), enabling binding of collectins, such as mannose-binding lectin (MBL). Free Sia can help to scavenge reactive oxygen group possibly by hydrolysis or structural alterations in Sia. βGal, beta galactosidase; BDNF, brain-derived neurotrophic factor; CCL21, CC-chemokine ligand 21; FH, complement factor H; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; NEU, neuraminidase, Neu5Ac, *N*-acetylneuraminic acid; polySia, polysialic acid; ROS, reactive oxygen species; SIGLECS, sialic acid-binding immunoglobulin-like lectins

The abnormal accumulation of Aβ and neurofibrillary tangles seen in AD are also associated with increased oxidative stress in the brain that is normally counterbalanced by microglial SIGLEC expression (Claude, Linnartz-Gerlach, Kudin, Kunz, & Neumann, 2013; Schwarz et al., 2015). In addition, oxidative stress seems to be a general contributor to aging, and not only to AD.

Thus, the Sia-complement axis as well as the Sia-SIGLEC axis and their fine-tuned regulation in both directions are of major interest for future therapy developments in age-related neurodegenerative diseases like AD.

Most Sias in the brain are found on sialoglycolipids called gangliosides (as reviewed in Schnaar et al., 2014). There are more than 60 known subtypes of gangliosides, differing in the position and number of Sias linked. The major ganglioside of the mammalian brain is GM1, with GD1a, GD1b, and GT1b expressed to a lesser degree (Schnaar et al., 2014). All four of these gangliosides share an identical tetra-saccharide core with varying amount of mono- or di-Sias attached to a Gal residue. Brain gangliosides have a variety of functions such as maintaining axon and myelin integrity (Sheikh et al., 1999) and regulating neuronal transmission (Schnaar et al., 2014; Takamiya et al., 1996). *Cis* interactions of gangliosides (on the same cell surface) are involved in the proper formation of lipid rafts, while *trans* interactions (between cell surfaces) may mediate binding to Sia-recognizing receptors like sialic acid-binding immunoglobulin-like lectins (SIGLECs).

PolySia is crucial for the development and function of the nervous system. It is involved in the regulation of synaptogenesis, neurogenesis, cell proliferation and migration, cell adhesion and axon guidance and modulates innate immune functions. Particularly, polySia plays an important role in learning and memory (as reviewed in Hildebrandt & Dityatev, 2013; Schnaar et al., 2014). Additionally, polySia binds and buffers neurotrophic factors, growth factors, neurotransmitters and cytokines (Sato & Kitajima, 2013; Sumida et al., 2015). For example, brain-derived neurotrophic factor (BDNF), which is locally produced and released in the CNS, is retained in the extracellular matrix bound to polySia under normal conditions

(Figure 2). However, under inflammatory conditions BDNF can be released via the removal of polySia on microglia by the sialidase NEU1 (Sumida et al., 2015). As mentioned before, polySia plays an important role during brain development. It is detectable from embryonic day 9.5 onwards and reaches peak level just before birth. After birth, polySia expression is strongly reduced in most brain regions, but is still detectable on synapses during adulthood (Sato & Kitajima, 2019), as well as in the neurogenic zones of the hippocampus and dental gyrus (K. Angata et al., 2004) and the whole retina (Karlstetter et al., 2017). α2,8-Linked polySia is mainly (85%) attached to neural cell adhesion molecule (NCAM; Sato & Kitajima, 2013) but is also found on other glycoconjugates such as synaptic cell-adhesion molecule (SynCAM; Galuska et al., 2010), neuropilin-2 (Curreli, Arany, Gerardy-Schahn, Mann, & Stamatos, 2007), and E-selectin ligand 1 (Thiesler, Beimdiek, & Hildebrandt, 2020; Werneburg et al., 2016). Polysialylated NCAM is present on microglia (Sumida et al., 2015), while polysialylated neuropilin-2 and E-selectin ligand 1 are restricted to the Golgi compartment of murine microglia and human THP-1 macrophages (Thiesler et al., 2020; Werneburg et al., 2016). Polysialylated neuropilin-2 and E-selectin ligand 1 can be translocated to the cell surface and released in the extracellular space by ectodomain shedding upon lipopolysaccharide (LPS) stimulation (Thiesler et al., 2020; Werneburg et al., 2016).

The sugar chains of cell surface glycoproteins and glycolipids, together with crosslinking proteoglycans, are collectively known as the glycocalyx, which forms a sugar coating and hydrophilic cloud over the surface of all mammalian cells (Figure 2). The terminal sugar residue of the glycoproteins and glycolipids is normally Sia, which constitutes the main negative charge of the cell surface. Sias from the glycocalyx can be recycled via uptake of sialoglycoconjugates. There is a continuous turnover of sialoglycoconjugates within lysosomes, releasing free sialic acid for reuse. The removal of Sias from the glycoconjugates is called desialylation and can be mediated by sialidases (as reviewed in Wei & Wang, 2019). Sialidases are glycoside hydrolase enzymes that remove terminal Sias from glycoproteins and glycolipids (Figure 1) and are the main mediators of desialylation (Schnaar et al., 2014; Wei & Wang, 2019). Humans and related mammals have several specific sialidases named neuraminidases (*N*-acetyl-alpha-neuraminidase 1–4; NEU1–NEU4; Pshezhetsky & Ashmarina, 2018; Wei & Wang, 2019). NEU1 is present at the plasma membrane and in the lysosome. NEU2 is found in the cytosol, NEU3 on the plasma membrane and NEU4 on internal membranes. Both NEU1 and NEU4 are active towards oligosaccharides, glycoproteins and gangliosides, while NEU1 mainly acts on glycoproteins and NEU3 shows preferential activity towards gangliosides (Pshezhetsky & Ashmarina, 2018). In addition, desialylation or loss of Sia recognition may be a consequence of Sias scavenging reactive oxygen species (Eguchi et al., 2002; Goswami, Nandakumar, Koner, Bobby, & Sen, 2003; Iijima, Takahashi, Namme, Ikegami, & Yamazaki, 2004). Due to their multiple hydroxyl-groups and high local concentration on the glycocalyx, Sias can be released and thereby scavenge reactive oxygen species (Figure 2), possibly leading to hydrolysis or structural alteration of Sias. Finally, Sias are spontaneously hydrolysed from the glycocalyx at a low rate, which is enhanced in an acidic environment (Manzi, Higa, Diaz, & Varki, 1994). Loss of Sia on proteins has been regarded as a sign of molecular aging, often leading to the removal of the underlying desialylated structure, for example mediating the turnover of blood cells and platelets (Grozovsky et al., 2014; Li et al., 2015; Mehdi et al., 2012; Qiu et al., 2016).

The presence of Sias has been shown to contribute to microdomain formation, cell adhesion, tissue homeostasis, cell migration, chemokine sensing, and growth factor retention (Bassagañas, Pérez-Garay, & Peracaula, 2014; Kelm, Schauer, Manuguerra, Gross, & Crocker, 1994; Kiermaier et al., 2016; Lübbers, Rodríguez, & van Kooyk, 2018; Möckl, Horst, Kolbe, Lindhorst, & Bräuchle, 2015; Sato & Kitajima, 2019; Varki & Gagneux, 2012). Furthermore, Sias are involved in the transport of micro- and macromolecules and can prevent receptor binding by masking underlying structures or the receptor itself (as reviewed in Schauer, 2000). Most of the observed effects of Sias may be related to its unique position as the terminal monosaccharide cap of the cell surface sugar chains and its negative carboxyl moiety with a pKa of 2.6 for Neu5Ac (Eylar, Madoff, Brody, & Oncley, 1962). Sias, especially polySias, contribute strongly to the negative charge and hydrophilic nature of the cell surface (Figure 2), and polySias bind and buffer soluble factors, including neurotrophins, growth factors and neurotransmitters (Sato & Kitajima, 2013). The affinity of proteins for sugar residues is normally relatively low, thus often requiring multiligand interactions between several sialoconjugates and the target structures (Cohen & Varki, 2014; Dam & Brewer, 2010). However, several receptors and other proteins of the innate immune system have been shown to specifically interact with cell-surface Sias, serving as a checkpoint for regulating the innate immune system. The mechanisms of this Sia sensing are discussed in more detail below.

3 | SIA SENSING BY THE COMPLEMENT SYSTEM

Sias can inhibit complement activation by modulating key functions of the complement system during immune surveillance and homeostasis. The complement system can be activated by three different pathways, the (a) classical, (b) lectin, and (c) alternative pathway (as reviewed in details by Ricklin, Hajishengallis, Yang, & Lambris, 2010). All three pathways have distinct initial triggers, but merge on C3 activation before triggering distinct effector pathways. For activation of C3, the inactive C3 protein is cleaved by a C3-convertase into the functional complement component 3a (C3a), complement component 3b (C3b) and other signaling fragments (e.g., iC3b). Whereas C3a attracts and activates immune cells, C3b (and its derivative the inactive form iC3b) acts as an opsonin to tag any nearby structures for phagocytosis (Merle, Church, Fremeaux-Bacchi, & Roumenina, 2015). In contrast to the classical and lectin pathway, the trigger of the alternative pathway is based on the spontaneous hydrolysis of an internal thioester bond of C3 that occurs continually at a low level during normal physiological conditions and initiates the mainly solvent-based C3-convertase

($C3_{H2O}Bb$) that cleaves C3 into C3a and C3b (Ricklin et al., 2010). Subsequently, C3b reacted with a foreign membrane can bind to cleaved complement factor B (Bb) to form a stable alternative pathway C3-convertase (C3bBb). As a consequence, the alternative pathway may contribute to the other two pathways by amplifying the cleavage and activation of C3 additionally and thus, appears to be involved in up to 90% of all complement activation events (Harboe & Mollnes, 2008). Therefore, the alternative pathway is highly regulated to avoid inappropriate damage to host tissues. The complement protein properdin can act on cell surfaces as initiator of the alternative pathway and in addition as an activator of C3 by stabilizing the alternative pathway C3-convertase (C3bBb) and forming a complex (C3bBbP). In contrast, the complement factor H (FH) competes with complement factor B (FB) to bind C3b in order to inhibit the formation of C3bBb and thus, negatively regulates the alternative pathway (Figure 3). FH also dissociates pre-existing C3-convertases (C3bBb) and attracts complement factor I (FI) to degrade C3b (Xue et al., 2017).

Recent research demonstrated that sialylation is critical for protecting fetal extraembryonic tissue from maternal complement attack involving C3 (Abeln et al., 2019). The exact mechanism for the fetal protective effect of Sias is still unclear and might be explained by several findings. In line with this protection, we recently showed that the absence of C3 rescued the synapse and neuronal loss in mice with reduced glycocalyx sialylation (Klaus et al., 2020). Free Sia has been shown to inhibit activation of C3 and the following complement cascade (Fujita et al., 1999). However, relatively high concentrations of free Sia were needed to observe this effect, questioning whether soluble Sia is responsible for the in vivo complement inhibition. More importantly, FH can bind Sias and is a well-known inhibitor of the alternative C3-convertase, as described before. In total, FH has at least three binding sites for polyanions such as Sias, located in the

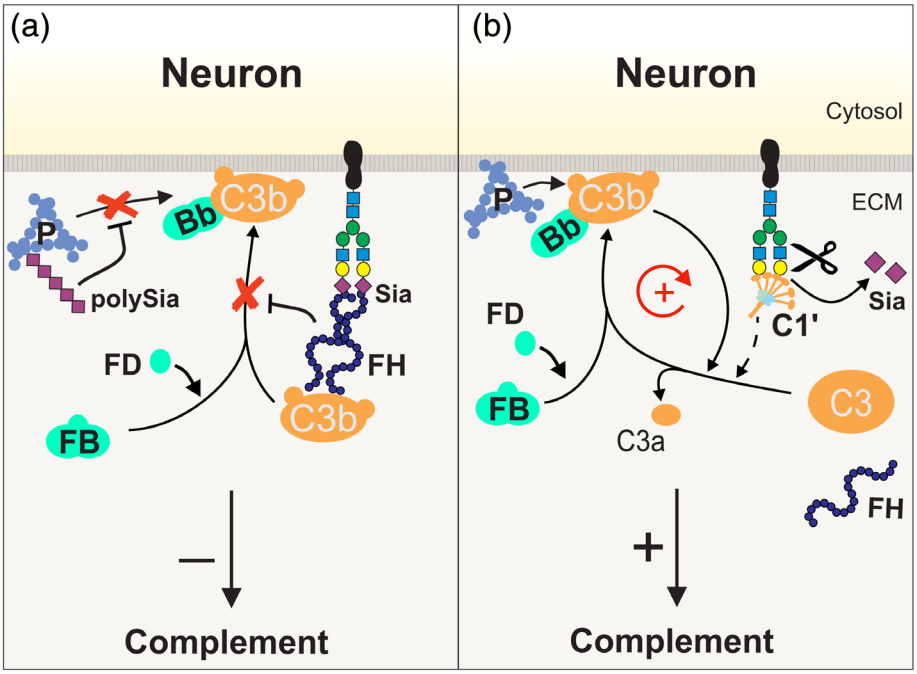

FIGURE 3 Sialic acid—complement interactions on intact versus altered glycocalyx. (a) Intact glycocalyx with normal sialylation: Under physiological conditions, the regulatory complement factor H (FH) can bind to sialic acids (Sia) of the host cell glycocalyx and competitively prevent binding of complement factor B (FB) to C3b. Thus, FH can inhibit the formation of the alternative C3-convertase (C3bBb) and consequently inhibits the activation of the alternative complement pathway on cells with an intact glycocalyx. Likewise, soluble polysialic acid (polySia) has been shown to inhibit the activation of the alternative complement pathway, for example, possibly via binding to the cationic protein properdin. (b) Altered glycocalyx with reduced sialylation: Properdin (P) binds to lesioned or altered cells (e.g., in the absence of sialic acid residues), that promotes C3b relocation to this site. It stabilizes the formation and activation of the alternative C3-convertase (C3bBb), together with the complement activator properdin (C3bBbP). Furthermore, lesioned or altered cells (e.g., in the absence of sialic acid residues) are opsonized by C1q, a subunit of the $C1qC1r_2C1s$ complex (C1). The "inactivated" C1 becomes activated (C1′) leading to a cascade with classical C3-convertase (C4b2b) activation, here only shown as dashed arrow. The classical C3-convertase then cleaves C3 into C3b and C3a. Weak binding of C3b on altered cells without FH binding could lead to further recruitment of FB activated by complement factor D (FD) and subsequently to the amplification of the complement pathway by the alternative C3-convertase (C3bBb). Thus, loss or removal of sialic acids (shown here as scissor) by neuraminidases, oxidative damage or aging triggers classical and alternative complement activation, facilitates C3b activation and the formation of the classical and alternative C3 convertases, which leads to an amplifying complement activation loop (red arrow). Bb, fragment of cleaved complement factor B; C1, $C1qC1r_2C1s$ complex; C3, complement component 3; C3a, fragment of cleaved complement component 3; C3b, fragment of cleaved complement component 3; ECM, extracellular matrix; FD, complement factor D; FB, complement factor B; FH, complement factor H; polySia, polysialic acid; P, properdin; Sia, sialic acid

region CCP 7, CCP 9–15, CCP 19–20, while the region CCP 19–20 was found to be key for FH interactions with sialylated host surfaces (summarized in detail by Ferreira, Pangburn, & Cortés, 2010). Importantly, FH only binds to α2,3-linked Sia residues of oligosaccharides typically attached to cell surfaces, but not soluble Sia monosaccharides (Schmidt et al., 2018). When FH binds to α2,3-linked Neu5Ac on the glycocalyx, it forms the active C3b–FH–Sia complex that inhibits the formation of the alternative C3-convertase (Figure 3a; Blaum et al., 2015). On the other hand, in the absence of Sia the underlying structures can be leading to classical complement pathway activation. Here, the desialylated cell surface structures are recognized by the complement complex C1 ($C1qC1r_2C1s$), triggering a complement cascade response via opsonization and formation of a C4b2b complex, the classical pathway C3-convertase. Again, absence of FH locally bound to cell surface sialic acid residues additionally could amplify the C3 activation via the alternative pathway C3-convertase (C3bBb). Thus, Sia residues of the glycocalyx are probably the critical determinants for classical and alternative complement pathway inhibition. Of note, the lectin pathway of complement activation also appears to be prevented by sialylation as Sias inhibit binding to cells of the mannose-binding lectin (MBL), the main initiator of the lectin pathway (Saevarsdottir, Vikingsdottir, & Valdimarsson, 2004).

FH gene variants can cause age-related macular degeneration (AMD), the main genetic cause of blindness (Ferreira et al., 2010). Two-year-old FH-deficient (*Cfh*−/−) mice showed an accumulation of complement C3 in the neural retina, together with a decrease in electron-dense material, thinning of Bruch's membrane, changes in the cellular distribution of retinal pigment epithelial cell organelles, and disorganization of rod photoreceptor outer segments (Coffey et al., 2007), but it is unclear how far this effect is related to Sia-binding of FH (Toomey, Kelly, Saban, & Bowes Rickman, 2015). Interestingly, aged FH-deficient mice also show typical features of AD in the retina with amyloid-β deposition and phosphorylated tau accumulation in the choroidal vessels associated with the retinal degeneration (Aboelnour, Kam, Elnasharty, Sayed-Ahmed, & Jeffery, 2016). Furthermore, FH appears to be a risk factor for AD in the Chinese population (Zhang et al., 2016). Mutations in the binding domain of FH prevents its ability to inactivate C3 by either disassembling the alternative pathway C3-convertase or supporting FI in its ability to degrade C3b (Ferreira et al., 2010). Similarly, mutations in complement receptor 1 (CR1, an AD risk gene, FI co-factor and complement regulator, Hourcade, Kathryn Liszewski, Krych-Goldberg, & Atkinson, 2000) enhanced binding of CR1 to C3b (Fonseca et al., 2016), which might increase microglial phagocytosis of synapses and neurons. Thus, there may be a link between dysfunctional complement regulation and AD (see Box 1, but an imbalance of Sias as regulators of the complement system was only recognized recently in the prodromal state of AD (Hajjar et al., 2020).

FH gene variants causing AMD potentially interfere with binding of FH to polyanions such as Sias, and thus allow uncontrolled complement deposition and activation followed by neurodegeneration, but this has not been verified. Recently we found that low molecular weight α2,8-linked polySia with an average degree of polymerization of 20 also attenuated alternative complement activation (Figure 3a), but not classical pathway activation, leading to reduced membrane attack complex formation and cell lysis in the retina (Karlstetter et al., 2017). Since FH binds to α2,3-linked Sia, but not α2,6- or α2,8-linked Sia (C. Q. Schmidt et al., 2018), the mechanism of this inhibitory effect of low molecular weight polySia on the alternative pathway remains elusive.

4 | SIA SENSING BY SIGLECS

SIGLECs belong to the class of I-type lectins that are characterized by three major components: (a) a set of 1–16 immunoglobulin domains which determine how far the N-terminal head group extends from the plasma membrane, (b) an extracellular carbohydrate recognition domain at the N-terminus which may recognize Sia residues on the glycocalyx of other cells (*trans*) or on the same cell (*cis*), and (c) an intracellular signaling tail. For some SIGLECs, SIGLEC-14, -15, -16 in humans and SIGLEC-3 and SIGLEC-H in mouse, the intracellular signaling tail contains a positively charged amino acid residue that can associate with activating adaptor proteins such as TYROBP/DAP12 bearing an immunoreceptor tyrosine activation motif (ITAM; Figure 4; Duan & Paulson, 2020). Upon ligand binding, such ITAM-containing adaptor proteins are recruited and become phosphorylated by a member of the spleen tyrosine kinase (SYK) family triggering a number of downstream signaling cascades similar as for other ITAM-SYK-signaling receptors like complement receptor 3 (CR3) or triggering receptor expressed on myeloid cells 2 (TREM2; as reviewed in Linnartz-Gerlach, Kopatz, & Neumann, 2014).

However, most SIGLEC receptors contain an intracellular immunoreceptor tyrosine-based inhibition motif (ITIM; Figure 4), which upon Sia binding lead to inhibitory downstream signaling pathways. Besides conserved SIGLEC-2, these SIGLECs include SIGLEC-E, -F and -G in mice and SIGLEC-3, -5, -6, -7, -8, -9, -10, -11, and -12 in humans (Duan & Paulson, 2020). Upon ligand binding to extracellular Sia residues, these SIGLECs containing intracellular ITIMs can counteract activatory signals emanating from receptors containing ITAMs (Crocker, Paulson, & Varki, 2007). The counteraction is initiated via phosphorylation of ITIMs by Src family kinases after ligand binding. The tyrosine-phosphorylated ITIMs then recruit tyrosine phosphatases, such as Src homology region 2 domain-containing phosphatase-1 (SHP-1/PTPN6) or Src homology region 2 domain-containing phosphatase-2 (SHP-2/PTPN11) (as reviewed in Duan & Paulson, 2020), which may dephosphorylate signaling molecules in the ITAM-signaling cascade of associated activatory receptors to suppress the activation of immune cells (Linnartz, Wang, & Neumann, 2010).

The ITAM-SYK-signaling pathway of TREM2/DAP12 or CR3/DAP12 (Figure 4b) induces phagocytosis in mononuclear phagocytes such as microglia. Multiple studies have shown how ITIM signaling of inhibitory SIGLECS could modulate this pathway. One study showed that SIGLEC-5 inhibited macrophage-mediated phagocytosis of apoptotic bodies upon binding of sialooligosaccharide ligands or

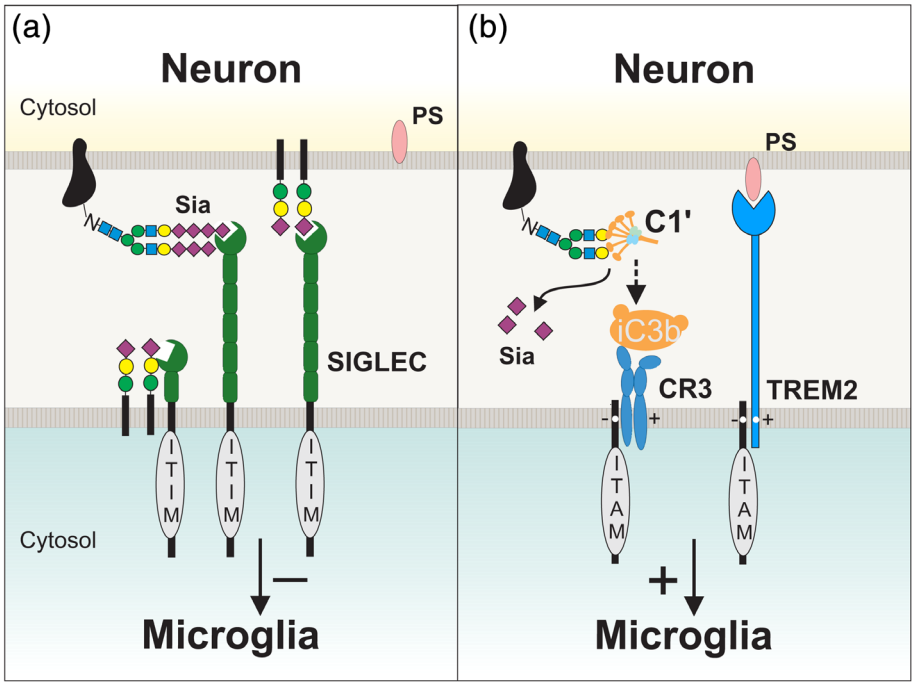

FIGURE 4 Sialic acid—SIGLEC interactions on intact versus altered glycocalyx. (a) Intact glycocalyx with normal sialylation: Sialic acids (Sia) on the intact glycocalyx can be sensed by SIGLEC receptors on the same cell (*cis*) and on other cells (*trans*). Inhibitory SIGLEC receptors such as SIGLEC-2/-3/-11 (human) and SIGLEC-E/-F (mouse) have an immunoreceptor tyrosine-based inhibition motif (ITIM) that upon ligand binding recruit phosphatases (such as SHP1/2) that can terminate intracellular signals emanating from immunoreceptor tyrosine-based activation motif (ITAM) signaling receptors via their intrinsic phosphatase activity. Thus, the ITIM signaling pathway inhibits several responses of microglia including phagocytosis, oxidative burst, migration, proliferation and inflammation and can down-regulate inflammasome activation. (b) Altered glycocalyx with reduced sialylation: Stressed or lesioned cells show desialylated glycoconjugates on their glycocalyx that can be opsonized by C1q within the C1 complex, which becomes activated (C1′). This initiates the formation of the C3-convertase that cleaves C3 into C3a, C3b and its downstream signaling fragment iC3b. The iC3b fragment binds to complement receptor (CR3) of microglia and activates the microglial cells via the ITAM of TYROBP/DAP12. Furthermore, other ITAM-signaling receptors of microglia such as TREM2 recognize aminophospholipids such as phosphatidylserine (PS) that are externalized and accessible after enzymatic removal or loss of the negatively charged sialic acids. The activatory receptors cannot be silenced by inhibitory SIGLEC receptors that fail to sense sialic acids on the altered glycocalyx of the lesioned cell. C1′, "activated" C1 complex; CR3, complement receptor 3 as heterodimer of CD11b/ITGAM and CD18/ITGB2; iC3b, soluble C3 signaling fragment; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; PS, phosphatidylserine; Sia, sialic acid; SIGLEC, sialic acid-binding immunoglobulin type lectin; TREM2, triggering receptor expressed on myeloid cells 2

monoclonal antibodies (Rapoport, Sapot'ko, Pazynina, Bojenko, & Bovin, 2005). Another study discovered that SIGLEC-11 was able to prevent microglial phagocytosis of apoptotic neuronal material and to attenuate LPS-induced inflammation (Wang & Neumann, 2010). In addition mouse SIGLEC-E prevented the removal of neural debris and the phagocytosis-associated oxidative burst of microglia (Claude et al., 2013). Interestingly, most inhibitory SIGLECs act as endocytic receptors after binding of small sized sialoglycoconjugates, while they inhibit phagocytosis of larger structures (Duan & Paulson, 2020). Beside inhibition of phagocytosis, the inhibitory ITIM-containing SIGLECs may also inactivate the inflammasome via inhibiting SYK signaling triggered by ITAM-signaling receptors. It is reported that both c-Jun NH2-terminal protein kinase (JNK) and SYK are necessary kinases for inflammasome activation (Linnartz & Neumann, 2013). Toll-like receptor (TLR) signaling contributes to the JNK kinase activation, whereas ITAM receptors stimulate SYK kinase. Thus, inhibitory SIGLECs may also prevent inflammatory activation of mononuclear phagocytes. There is evidence in B-cells that ligand binding sequester inhibitory SIGLECs away from activatory receptors, thus increasing the signaling effect of the activatory receptors (Duan & Paulson, 2020). However, so far this phenomenon has not been observed for microglial cells.

SIGLECs are mainly expressed on immune cells, such as microglia, macrophages and monocytes with a few exceptions, such as for SIGLEC-4 (expressed in oligodendrocytes and Schwann cells) and SIGLEC-6 (also expressed in placental trophoblasts) as nicely summarized by Duan and Paulson (2020). All inhibitory SIGLECs expressed on microglia including mouse SIGLEC-2, human SIGLEC-3, SIGLEC-11, mouse SIGLEC-E, and SIGLEC-F have *trans* interaction with Sia residues on neighboring cells and several SIGLECs including SIGLEC-3 and SIGLEC-E have been demonstrated to also bind Sia residues in *cis* (Figure 4a; Bornhöfft, Goldammer, Rebl, & Galuska, 2018).

The first functional studies on human *SIGLEC-11* were carried out by ectopic expression of this human receptor on murine microglia (Wang & Neumann, 2010). The expression of SIGLEC-11 was able to

inhibit the proinflammatory response of LPS-challenged microglia and prevented microglial phagocytosis of apoptotic neuronal material. This neuroprotective effect of SIGLEC-11 was mediated by *trans* interaction with polySia on neighboring neurons, but not by *cis* interaction with its own glycocalyx (Wang & Neumann, 2010). Similar neuroprotective effects were shown for SIGLEC-E (Claude et al., 2013). SIGLEC-E inhibited the phagocytosis of neural debris. Furthermore, SIGLEC-E on microglia prevented the neural debris-triggered release of superoxide and production of proinflammatory cytokines. Although on a low level *cis* interaction can be found for SIGLEC-E, our data indicated that the neuroprotective role of SIGLEC-E was mediated in *trans* via recognition of Sia residues on neurons (Claude et al., 2013). Interestingly, the release of the microglia-intrinsic polySia pool from Golgi upon inflammatory stimulation acted as a *trans*-activating ligand of SIGLEC-E, thus inhibiting the inflammatory response in traumatic brain injury (Thiesler et al., 2020). In contrast, CRISPR/Cas9-mediated *Siglec-E* knockout of the murine microglial cell line BV2 resulted in a more pronounced increase of LPS-induced proinflammatory cytokines on transcription level. Furthermore, *Siglec-E* knockout of BV2 prevented the anti-inflammatory effect of exogenously added polySia (Thiesler et al., 2020).

Both neurodegeneration and aging have been associated with dysfunction or deletion of SIGLEC receptors (Duan & Paulson, 2020; Schwarz et al., 2015). SIGLEC receptors on microglial cells interact with Sia to inhibit microglial activation, inflammation, phagocytosis and oxidative burst (Figure 4a). *Siglec-E* KO mice showed oxidative damage to cellular DNA, proteins and lipids in all organs that was related both to an unbalanced ROS metabolism, and to a secondary impairment in detoxification of reactive molecules (Schwarz et al., 2015). Consequently, *Siglec-E* KO mice exhibited accelerated aging and their life span was reduced (Schwarz et al., 2015). Interestingly, an opposite phenotype was observed after deletion of the crucial activatory microglial TREM2 receptor (Linnartz-Gerlach et al., 2018). Deletion of the main counter-balancing activatory TREM2 receptor in mice led to decreased age-related inflammatory signs and reduced neuronal loss in the substantia nigra and the hippocampus of 24 months old mice (Linnartz-Gerlach et al., 2018). The absence of age-related neurodegeneration in *Trem2* KO mice was associated with decreased microglial numbers and less accumulation of oxidized lipids (Linnartz-Gerlach et al., 2018).

Meanwhile, it also has been shown that human SIGLEC-3 prevents microglial uptake of amyloid-β 42 (Aβ42) (Griciuc et al., 2013). Human SIGLEC-3, also known as CD33, is abundantly expressed on microglia. In contrast to other SIGLECs, SIGLEC-3 is capable to interact with Sia in both *trans* and *cis*. Two splice variants of human CD33 are relevant for AD: the full-length CD33M isoform and the shorter CD33m isoform, which lacks the exon 2-encoded Sia ligand-binding domain (Malik et al., 2013). Several genome-wide association studies (GWAS) indicated that the full length CD33M is a risk factor for AD, whereas a decreased AD risk was associated with the shorter CD33m isoform (Malik et al., 2013). Furthermore, increased expression of CD33M was detected in microglial cells of AD brains, further supporting the GWAS findings (Hollingworth et al., 2011; Naj et al., 2011). Interestingly, the phagocytosis of Aβ42 by microglia was inhibited with increasing human CD33M levels. Concomitantly, the Aβ pathology was attenuated in amyloid precursor protein (APP)-transgenic mice by inactivating CD33, and thus possibly increasing the uptake of Aβ42 (Bradshaw et al., 2013; Griciuc et al., 2013). These data suggest that any weakening of the Sia-SIGLEC-3 axis, either by desialylation or variants of SIGLEC-3/CD33m lacking the Sia-binding domain might increase microglial phagocytosis of Aβ42, and hence potentially could be beneficial in AD pathology (see Box 1). Importantly, the human and the mouse SIGLEC-3 share the same name but not the exact same function. While the human SIGLEC-3 is an inhibitory receptor with an ITIM domain, the mouse SIGLEC-3 has only an ITIM-like domain. Consequently, both receptors fulfill different functions as shown previously (Bhattacherjee et al., 2019). While the human SIGLEC-3 inhibits monocyte and microglial phagocytosis, no effect on phagocytosis was found for the mouse SIGLEC-3 (Bhattacherjee et al., 2019).

Most recently, it was reported that SIGLEC-2, also called CD22, downregulates microglial phagocytic ability during aging and SIGLEC-2 blockade promotes removal of α-synuclein fibrils, Aβ oligomers and myelin debris in vivo (Pluvinage et al., 2019). Furthermore, SIGLEC-2 was upregulated in microglia during aging (Pluvinage et al., 2019), and in the brains of AD patients (Friedman et al., 2018). By activating CD22 with synthetic glycopolymers bearing α2–6-linked Sia, it was shown that SIGLEC-2 on microglia inhibited phagocytosis (Pluvinage et al., 2019). Interestingly, inhibition of SIGLEC-2 with SIGLEC-2 blocking antibody or genetic ablation showed the opposite effect and increased the phagocytosis of Aβ oligomers, myelin debris and α-synuclein fibrils in vivo. Long-term blockade of SIGLEC-2 using blocking antibody of SIGLEC-2 via CNS-delivery was even able to restore microglial homeostasis and to attenuate the cognitive decline of aged mice (Pluvinage et al., 2019).

5 | EFFECTS OF HYPERSIALYLATION OR DECREASED NEURAMINIDASE ACTIVITY

Cancer cells often misuse Sias as checkpoint to escape the immune control. Therefore, cancer cells typically have increased expression of different sialyltransferases, leading to increased Sia levels and increased incorporation of sialoconjugates into the glycocalyx of their cell membrane (Duan & Paulson, 2020; Rodrigues & Macauley, 2018).

Interestingly, some bacteria can exploit this self-recognition system by incorporation of Sias into their own capsule, thus masking their antigenic cell surface and escape the immune surveillance. They may acquire Sia via sialidase-mediated removal from host tissue or via de novo generation of Sias as molecular camouflage to escape immune defense. Several bacteria also produce polySia by themselves and thus escape host immune attack via inactivating complement system and silencing innate immune cells via SIGLEC-11. For example, the polySia capsule of *Escherichia coli* K1 (*E. coli* K1) inhibits alternative complement pathway activation (Pluschke, Mayden, Achtman, &

Levine, 1983) and engages the inhibitor receptor SIGLEC-11 (Schwarz et al., 2017), while bacteria of serogroup B *Neisseria meningitides* use α2,8-linked polySia overexpression to escape elimination by antibody-dependent classical complement attack in normal human serum (Kahler et al., 1998).

Impaired or insufficient activity of neuraminidases can be harmful for the cell. For example, NEU3 and NEU4 are important enzymes in the lysosomal degradation of gangliosides by removing the Sia residues (Figure 5). The deficiency of *Neu3* and *Neu4* in mice resulted in the accumulation of nondigested ganglioside GM3 in lysosomes of microglia, vascular pericytes and neurons. Furthermore, this impaired lysosomal storage resulted in micro- and astrogliosis, neuroinflammation, and accumulation of lipofuscin bodies in the mouse brain (Pan et al., 2017). Thus, the cleavage of terminal Sias is essential in ganglioside catabolism. Moreover, it is well-known that neuraminidase deficiencies within the lysosome leads to accumulation of undegraded sialoconjugate substrates, leading to neurodegenerative lysosomal storage diseases (Figure 5). For example, genetic deficiency of *NEU1* in humans causes sialidosis Type 1 and 2 (Pshezhetsky & Ashmarina, 2018), which triggers severe pathologies in the central nervous system including retinopathy, ataxia, mental retardation, myoclonus and seizures (Seyrantepe et al., 2003). NEU1 deficiency induces over-sialylation of lysosomal-associated membrane protein 1 (LAMP1) that is leading to excessive lysosomal exocytosis (Yogalingam et al., 2008). Thus, reduced neuraminidase activity causes both, accumulation of sialylated lipids and proteins in lysosomes, visible as lipofuscin in neurons and lipid drops in microglia/macrophages, as well as increased expelled sialylated glycoconjugates that appear as extracellular protein aggregates or lipid drops (Figure 5).

Of note, APP is normally sialylated and can be desialylated by NEU1. In the *Neu1*-deficient mouse, which has impaired desialylation, hypersialylated APP accumulated in the lysosomes of neural tissue. In

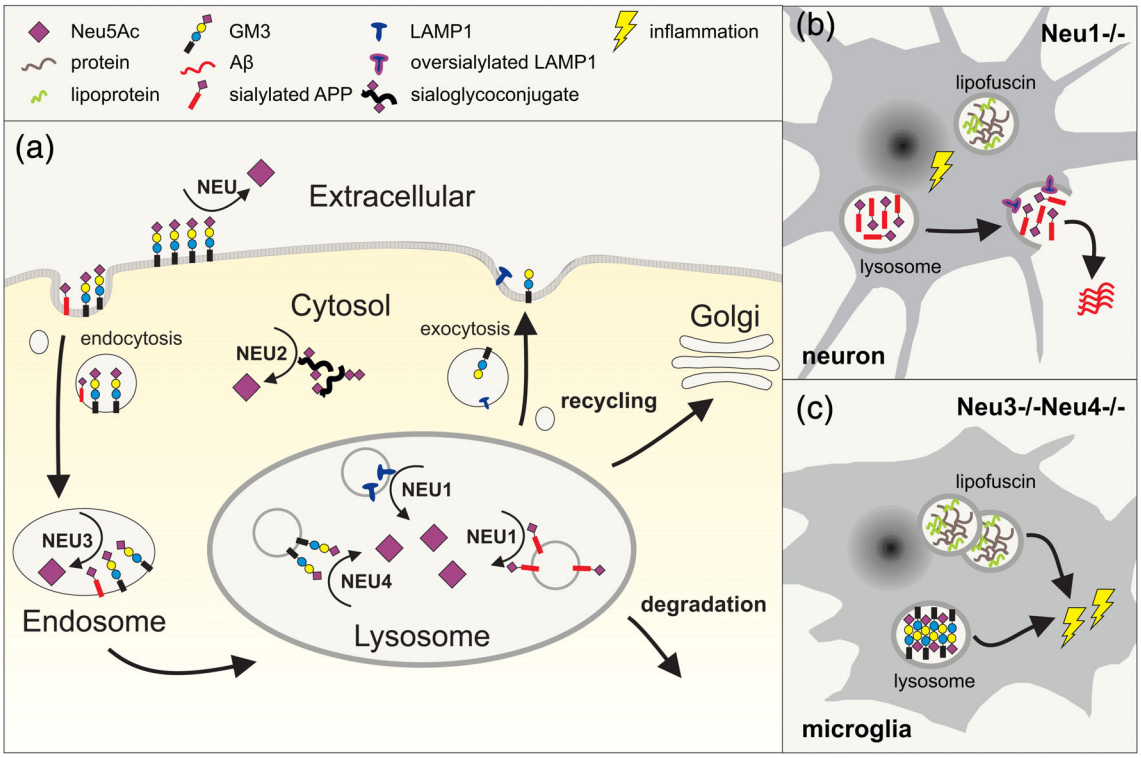

FIGURE 5 Decreased neuraminidase activity leads to lysosomal and extracellular protein and lipid accumulation. (a) The removal of sialic acids is catalyzed by neuraminidases (NEUs). The degradation of sialoglycoconjugates is an essential step for the reuse or digestion of the lipids and proteins. In humans four NEUs are known NEU1–4. NEU1–4 show tissue- and compartment-specific preferences and also substrate specificity. NEU1 is mainly localized at the lysosomal and plasma membranes, whereas NEU2 is mainly found in the cytosol. NEU3 is localized in the caveolae microdomains or plasma and endosomal membranes, while NEU4 is predominately present in the endoplasmic reticulum membranes and in the lysosome as well as the mitochondria. Desialylation greatly influences the structure of glycoconjugates and is creating or changing their binding sites. Furthermore, NEUs contribute to the regulation of various cellular functions, such as lysosomal degradation or exocytosis. For example, neuraminidase 1 (NEU1) negatively regulates lysosomal exocytosis of by-products by processing the sialic acid residues on the lysosomal-associated membrane protein 1 (LAMP1). (b) Deficiency in NEU1 (NEU1−/−) is leading to accumulation of sialylated lipids and proteins in the lysosome, visible as lysosomal swelling or lipofuscin accumulation. In Neu1−/− mice, oversialylated amyloid precursor protein (APP) was accumulated in the lysosomes of neurons. Furthermore, the deficiency in removal of sialic acid residues from the lysosomal protein LAMP1 increased the exocytosis of undegraded proteins like APP to the extracellular space, thus, supporting Aβ aggregation. (c) NEU3 and NEU4 are very important in the processing and degradation of gangliosides in the central nervous system. In NEU3/NEU4-deficient mice (Neu3−/-Neu4−/−), the ganglioside GM3 was not properly degraded and was accumulated in lysosomes of microglia, vascular pericytes and neurons. This resulted not only in decreased levels of gangliosides, but also in micro- and astrogliosis, cognitive impairment and lipofuscin body accumulation

an AD mouse model overexpressing the human mutant APP an additional ablation of *Neu1* accelerated Aβ production that was leading to excessive lysosomal exocytosis of amyloid-β peptides into the extracellular space (Annunziata et al., 2013). Plaque load was reversed in this mouse model by enforced overexpression of *NEU1* via injection of an adeno-associated virus (AAV) containing the human *NEU1* (Annunziata et al., 2013). Hence, reduced activity or capacity of *NEU1* to digest sialoconjugates in the lysosome may be a risk factor for developing aggregopathies such as AD.

Interestingly, not only the accumulation of sialoconjugates in lysosomes but also in endocytic compartments can impair the degradation capacity of the lysosomal system (Schmid, Mach, Paschke, & Glössl, 1999). Accordingly, the bacteria *Pseudomonas aeruginosa* uses Sia to indirectly inhibit phagosome maturation in macrophages. Although the mechanism is not fully understood, the phagosome maturation is probably impaired by sialoconjugates due to reduced phagosome-lysosome fusion (Mukherjee, Khatua, & Mandal, 2020). Since the lysosome plays an important role in the degradation and recycling of extra- and intracellular substances, impaired removal of Sias and thus accumulation of misfolded proteins might be a potential target in treating lysosomal storage diseases.

In addition, the composition of Sias is markedly different between humans and other mammals. As mentioned before, humans can only produce Sias from Neu5Ac due to a loss-of-function mutation in the enzyme cytidine monophospho-*N*-acetylneuraminic acid hydroxylase (CMAH), while most other mammals including mice produce and express Neu5Ac and Neu5Gc (Altman & Gagneux, 2019). It is still unclear whether there is any contribution of the nonhuman Sia type Neu5Gc to neuroinflammatory or neurodegenerative disease, but trace amounts of Neu5Gc were found in human tissue, probably derived from consumed red meat and dairy products (Banda, Gregg, Chow, Varki, & Varki, 2012; Casadesús et al., 2013). This nonhuman Sia type can trigger autoantibodies in humans, resulting in the autoimmunity termed "xenosialitis" (Dhar, Sasmal, & Varki, 2019; Varki, 2017). Neu5Gc cannot be digested properly by the human endogenous neuraminidases after incorporation into α2-8-linked oligo-/polySias (Davies et al., 2012) and this can result in hypersialylation or impaired desialylation.

While insufficient activity or capacity of neuraminidases to digest sialoconjugates in the lysosome might trigger or promote accumulation of lipid and protein aggregates as seen in AD, none of these signs were observed in human diseases associated with hypersialylation such as cancer (Rodrigues & Macauley, 2018).

6 | EFFECTS OF DESIALYLATION OR INCREASED NEURAMINIDASE ACTIVITY

The removal of the negatively charged Sias by desialylation regulates multiple cellular functions and pathological processes (Wei & Wang, 2019). In particular, desialylation regulates brain innate immunity and microglial homeostatic function. We found that various inflammatory stimuli such as LPS, fibrillar Aβ, or Tau induced a sialidase activity on the surface of microglial cells, desialylating surface glycans and maintaining microglial activation in culture (Allendorf, Puigdellívol, & Brown, 2020). In vivo, exposure of postnatal rats to LPS induced neuraminidase activity and a long-lasting desialylation of glycoproteins on neural cells in the CNS (Demina et al., 2018). Furthermore, desialylation in postnatal rat brains was associated with a primed microglial phenotype and thus might have pathophysiological consequences in adulthood.

Sialylation of neurons also acts homeostatically on neighboring cells via SIGLECs (Figure 6). For instance, interaction between gangliosides and SIGLEC-4 (MAG, myelin associated glycoprotein) mediates myelin stability and inhibits neural sprouting after injury. Consequently, spinal axon outgrowth in rat was enhanced after removal of Sias by sialidases (Mountney et al., 2010). Furthermore, desialylation can enhance microglial phagocytosis of neuronal structures via decreased activation of inhibitory SIGLECS, including SIGLEC-2 (CD22; Pluvinage et al., 2019), SIGLEC-3 (CD33; Bradshaw et al., 2013), SIGLEC-11 (Wang & Neumann, 2010), and SIGLEC-E (Claude et al., 2013).

Cell surface receptors can be regulated by sialylation and desialylation of the receptors themselves (Figure 6). Attachment or detachment of the negatively charged Sias can affect receptor binding, receptor activity or receptor turnover (Pshezhetsky & Ashmarina, 2018). Several receptors regulating microglial activation and phagocytosis are stimulated by desialylation, suggesting the possibility that sialylation of microglial receptors acts as a general "OFF" switch or immune checkpoint, while desialylation licenses these immune receptors to operate. Microglial TLR4 was found to be activated after exposure to sialidases in vitro and in vivo (Allendorf, Franssen, & Brown, 2020; Fernández-Arjona, Grondona, Fernández-Llebrez, & López-Ávalos, 2019). We found that activation of microglia by LPS caused Neu1 to translocate to the cell surface and to desialylate TLR4 (Figure 6). Furthermore, microglial activation was maintained even after LPS was removed, constituting a type of immune memory (Allendorf, Franssen, et al., 2020). The inflammatory effects of TLRs can be down-regulated by SIGLECs (Chen et al., 2014). We could show that SIGLEC-E inhibited microglial activation by binding sialylated TLR4, and this inflammatory break was released by desialylation of TLR4 (Allendorf, Franssen, et al., 2020). Activation of microglia with LPS was also found to cause Neu1-mediated removal of microglial polySia, leading to release of BDNF normally bound to polySia (Sumida et al., 2015).

Another important microglial receptor class are integrins that mediate cell adhesion and phagocytosis. Desialylation of the fibronectin receptor on myeloid cells increased fibronectin binding many-fold by exposing the binding site (Pretzlaff, Xue, & Rowin, 2000; Semel et al., 2002). Desialylation of the integrin CR3 increased the binding to its ligand intercellular adhesion molecule 1 (ICAM-1; Feng et al., 2011). Moreover, we found that sialidases induced microglial phagocytosis via CR3 (Allendorf, Puigdellívol, et al., 2020). However, it is unclear whether microglial CR3 is directly regulated by sialylation or indirectly via SIGLEC-E binding as occurs in neutrophils (McMillan et al., 2013).

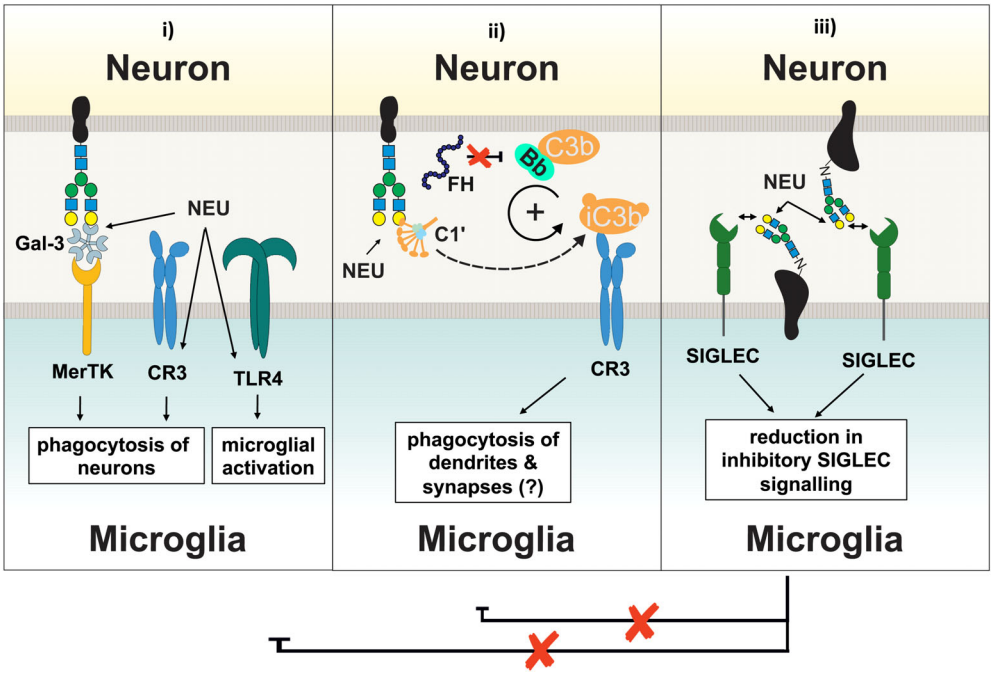

FIGURE 6 Effects of desialylation on microglial activation and phagocytosis of neurons. Cell surface receptors can be regulated by sialylation and desialylation. Inflammatory activation of microglia induces extracellular neuraminidases (NEU) that desialylates multiple targets. (a) Neuronal desialylation promotes binding of galectin-3 (Gal-3), which stimulates microglial phagocytosis of neurons via c-mer proto-oncogene tyrosine kinase (MerTK). Furthermore, microglial desialylation induces activation of complement receptor 3 (CR3) and toll-like receptor 4 (TLR4), promoting phagocytosis of neurons and microglial activation. (b) Desialylation of dendrites increases binding of the "activated" complement complex C1 (C1′) to dendrites, which initiates the classical complement pathway with formation of the classical C3-convertase (dashed arrow), that is leading to cleavage of C3 into C3a and C3b, as well as iC3b. The signaling fragment iC3b activates microglial phagocytosis via complement receptor 3 (CR3). Removal of surface sialic acid also reduces binding of the regulatory complement factor H (FH), thus enhancing formation of alternative C3-convertase (C3bBb), that is also leading to cleavage of C3 into C3a and C3b, as well as iC3b. C1′ deposition at the synapse is probably also mediated by desialylation. (c) Desialylation of neurons and microglia decreases *cis* and *trans* sialic acid binding immunoglobulin-like lectin (SIGLEC) binding, thus reducing the homeostatic inhibitory signaling that normally counteracts the phagocytosis pathways in microglia (a and b). Bb, fragment of cleaved complement factor B; C1′, "activated" C1qC1r$_2$C1s complex; C3b, fragment of cleaved complement component 3; CR3, complement receptor 3; FH, complement factor H; iC3b, soluble C3 signaling fragment; NEU, neuraminidase; MerTK, Mer Tyrosine Kinase; Gal-3, galectin-3; TLR4, toll-like receptor 4; SIGLEC, sialic acid binding immunoglobulin-like lectin

Desialylation of cells enables binding of the galactose-binding opsonin galactin-3 (Nomura, Vilalta, Allendorf, Hornik, & Brown, 2017). Galectin-3 can be released by LPS-stimulated microglia (Burguillos et al., 2015), together with a sialidase (probably NEU1), which can desialylate neighboring cells (Nomura et al., 2017; Figure 6). When desialylation of neurons occurs, galectin-3 binds to uncovered Gal residues of glycoproteins and glycolipids on neurons, that normally are occupied by terminal Sias (Figure 2). This galectin-3 binding enhanced the phagocytosis of neuronal-like cells by microglia (Nomura et al., 2017). Note that galectin-3 can activate microglial TLR4 (Burguillos et al., 2015; Yip et al., 2017), c-mer proto-oncogene tyrosine kinase (MerTK; Nomura et al., 2017) and TREM2 (Boza-Serrano et al., 2019), but it is not known whether galectin-3 is specifically binding the desialylated forms of these receptors. Desialylation of cells also enables the binding of C1q (Linnartz, Kopatz, Tenner, & Neumann, 2012), calreticulin (Feng et al., 2018) and mannose binding lectin (Saevarsdottir et al., 2004), which can promote phagocytosis and complement activation.

As indicated above, Sias inhibit the complement cascade, indicating that desialylation could activate the complement system (Figure 6). In vivo, rats treated with neuraminidase were found to have C3 activation in cerebrospinal fluid, followed by ependymal damage. Ependymal cell death was provoked after the neuraminidase-mediated removal of the protective Sia levels from the cell surfaces (Granados-Durán et al., 2015. in vitro studies indicated that the removal of neurites by microglia could be induced by desialylation of neurons or microglia (Allendorf, Puigdellívol, et al., 2020; Linnartz et al., 2012; Linnartz-Gerlach, Schuy, Shahraz, Tenner, & Neumann, 2016). When neurons were desialylated, C1q (a subunit of the C1 complex) and C3 bound to neurites, resulting in CR3-mediated microglial phagocytosis of the neurites (Figure 6; Linnartz-Gerlach et al., 2016; Linnartz et al., 2012). It is not clear why C1q binds more to desialylated neurons, but this might be mediated by C1q binding to pentraxin-3, as it has been shown that desialylation of pentraxin-3 allows C1q binding and complement activation (Inforzato et al., 2006). We also found that desialylation of microglia triggered CR3-mediated phagocytosis of neurons in a co-culture system (Allendorf, Puigdellívol, et al., 2020).

GNE is required for Sia synthesis, and homozygous knockout of *Gne* in mice is embryonically lethal (Schwarzkopf et al., 2002), indicating the

essential roles of Sias. Recently, we analyzed the effect of partial desialylation by using viable heterozygous *Gne* knockout mice with a mild (~20–30%) reduction in sialylation (Klaus et al., 2020). We discovered that microglia were activated at 6–9 months of age with reduced arborization and reduced synapse numbers in the heterozygous *Gne* knockout mice. In parallel, a gradually increased neuronal loss in the brain was found at 12 months of age that was not present in the wildtype control mice. Interestingly, by crossbreeding these mice with complement *C3*-deficient mice, we were able to restore the loss of neurons and synapses, as well as the reduced microglial arborization, confirming the complement C3-dependent removal of desialylated structures (Klaus et al., 2020). Of note, synapse loss is also found in different neurodegenerative diseases, including AD and it was demonstrated that the knockout of complement proteins C1q, C3, or CR3 is able to prevent synaptic and memory loss in animal models of these diseases (Schafer & Stevens, 2010). In summary, these data suggest that the sialylation status in the "Sia-complement" axis plays a critical role in modulating microglial phagocytosis in both aging and neurodegenerative diseases.

In humans, mutations in the *GNE* gene lead to a group of genetic disorders known as "GNE myopathy." The main clinical features are progressive muscle atrophy and weakness leading to wheelchair-bound patients, while the histopathology shows desialylation of specific muscle proteins and "rimmed" vacuole inclusions in the affected muscle tissue. Interestingly, abnormal accumulations of Aβ protein, ubiquitin, and phosphorylated tau protein, accompanied by cell stress, can be found in some of the degenerating muscle fibers (Argov & Yarom, 1984; Murakami et al., 1995; Muth et al., 2009; J. Schmidt et al., 2012), but no protein aggregates have so far been described in the CNS of these patients. Similar findings were seen in transgenic mouse models bearing the human GNE D176V (Anada et al., 2014; Cho et al., 2017; Malicdan, Noguchi, Nonaka, Hayashi, & Nishino, 2007) or the human GNE M712T mutation (Galeano et al., 2007), but the mechanism leading to protein aggregation in muscle tissue is not fully understood. However, it has been proposed that the protein aggregation seen in GNE myopathy might share some common pathogenic mechanisms with the accumulation of Aβ protein in the AD brain.

In summary, sialylation of CNS cells appears to inhibit microglial activation and phagocytosis by (a) blocking complement C3 activation and opsonization, (b) blocking binding of the opsonin and alarmin galectin-3, (c) inhibiting some key innate immune receptors such as TLR4, and (d) activating inhibitory SIGLEC receptors. Thereby, sialylation can be seen as a general "OFF" switch or immune checkpoint, while desialylation removes this regulating checkpoint.

7 | CONCLUSIONS

Sias keep the CNS innate immune response in homeostasis by inhibiting complement, as well as microglia via SIGLECs and other receptors. Interestingly, the level of Sias is strictly regulated in the CNS environment (cell type, subcellular structure and individual protein) and time (development, aging, and inflammation), allowing local and conditional responses without changing the overall activation profile of cells. Thus, the Sia checkpoint is a promising therapeutic target to treat human neuroinflammation and neurodegeneration. However, it is still unclear which disease types and at which disease stage the inhibitory effects of Sias should be strengthened or weakened. While some data indicate that a weakening of this axis for example might help to clear Aβ plaques in AD animal models, the overall concept suggests strengthening the inhibitory effect of Sias to avoid excessive inflammatory, complement and radical-mediated damage of postmitotic neuronal cells. In particular, desialylation triggers microglial activation, oxidative damage, microglial phagocytosis of neurons and accumulation of oxidized lipids and proteins. Likewise, failure of neuraminidases to digest sialoconjugates also triggers accumulation of lipids and protein aggregates. Thus, more data and a better understanding of the fine-tuning of this crucial Sia checkpoint are urgently required.

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CONFLICT OF INTEREST

H. N. is named inventor on a patent related to the therapy of neurodegenerative diseases by polySia (patent family to WO2014154537A1) that is assigned to his employer. C. K., H. L., D. H. A., and G. B. declare no conflict of interest.

AUTHOR CONTRIBUTIONS

C. K., H. L., D. H. A., G. B., and H. N. wrote the manuscript and designed the figures.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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